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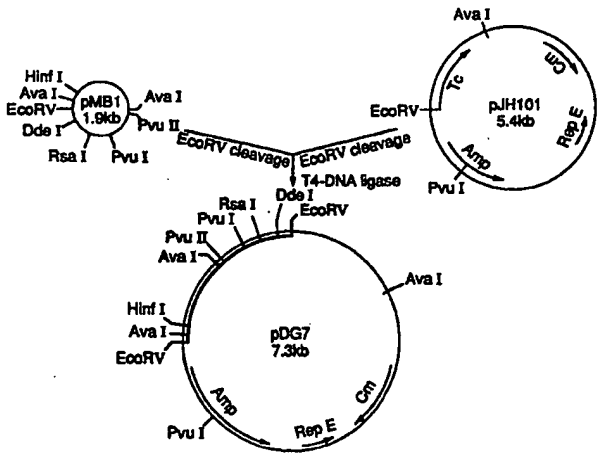
(54) Title: METHOD FOR INTRODUCTION OF GENETIC MATERIAL INTO MICROORGANISMS AND TRANSFORMANTS OBTAINED THEREWITH




(57) Abstract

A method of introducing nucleic acid into microorganisms and microorganisms transformed by such method. Known methods for transforming microorganisms to provide them with new genetic information have limited applicability. Some microorganisms are difficult to transform or transfect by the known techniques. These microorganisms are called recalcitrants. Some lactic acid bacteria, especially *Lactobacillus* and *Bifidobacterium* species are difficult to transform by known techniques. The invention solves this problem by subjecting the microorganism to limited autolysis in or before the transformation process. Said limited autolysis is carried out by incubating the microorganism in low molarity electroporation buffer containing an osmotic stabilizer, generally at a pH between 4 and 8, at a temperature below 37 °C, such as between 0 and 10 °C. The invention provides criteria for selecting plasmids to transform the microorganism with, and suitable plasmids for transformation. Applications of the transformed microorganisms are also disclosed.

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Title: Method for introduction of genetic material into microorganisms and transformants obtained therewith

Field of the invention

The invention lies in the field of genetic engineering or modification using recombinant DNA technology and relates in particular to the genetic modification of microorganisms, in particular Lactic Acid Bacteria, more specifically to bacteria of the genus *Bifidobacterium*.

The invention relates to methods for introducing nucleic acid into said microorganisms, to plasmids that can be employed for transforming said microorganisms, to markers that can be used to select for transformed microorganisms, to transformants which are obtainable by the previously mentioned methods and to products that can be obtained through expression of genes present on plasmid vectors in said microorganisms.

The invention particularly relates to such methods for introducing nucleic acid into microorganisms which can hardly be transformed or transfected by the methods existing in the state of the art. These microorganisms are often referred to (and will be hereinunder) as "recalcitrants". Some lactic acid bacteria, especially some *Lactobacillus* and *Bifidobacterium* species qualify as recalcitrants. The invention specifically relates to methods for introducing nucleic acids into these organisms and to the organisms obtainable thereby.

Furthermore the invention relates to applications of said microorganisms.

Background of the invention

Bifidobacteria constitute one quarter of the gut flora of normal healthy adults (H.M. Modler, R.C. McKeller and M. Yaguchi (1990) Can. Inst. Food Sci. Technol. J. 23(1), 29-41). These bacteria are thought to have beneficial properties for their host. The use of bifidobacteria as starter cultures for the preparation of yoghurt and other fermented milk products thus may help in promoting health. The genera *Bifidobacterium* and *Lactobacillus* were recognized for many years as genuine members

of the Lactic Acid Bacteria (LAB) group. According to more recent views the two genera are classified in two different families. LAB are Gram-positive, anaerobic, micro-aerophilic, catalase-negative rods or cocci (genus *Lactococcus*), including the genera *Lactobacillus, Lactococcus, Pediococcus, Streptococcus, Leuconostoc, Enterococcus* and *Carnobacterium;* most importantly, they all produce lactic acid as an important end product from the energy yielding fermentation of sugars. Besides the health promoting properties of some *Bifidobacterium* and *Lactobacillus* species for humans, the economic importance of these microorganisms is beyond doubt, because they are widely used as starter cultures for the production of feed- and food products, especially dairy products.

To allow a more complete exploitation of the potential of these organisms for practical applications, detailed knowledge is required about such basic biological phenomena as cellular metabolism, gene expression, protein secretion etc. The availability of recombinant DNA techniques also allows to carry out strain improvement programs in a more directed and faster way than is possible using the conventional "mutation and selection" methodology.

The present invention relates to the development of a system for the efficient and reproducible genetic transformation of microorganisms, especially of "recalcitrant strains" of bacteria. Recalcitrant bacteria are defined herein as bacterial strains that cannot be transformed, or with great difficulty only, by any of the common transformation protocols used by persons skilled in the art. Commonly used transformation techniques involve the introduction of DNA in bacteria, i) after rendering the bacteria transformation competent, ii) after protoplast formation of the bacteria, or iii) by various electroporation procedures.

The present invention relates in particular to a procedure for transforming bacteria of specific genera of LAB, such as for example bacteria of the genus *Bifidobacterium*, or of specific strains of LAB genera. For many *Lactobacillus* species useful transformation procedures are available. However, some *Lactobacillus* strains (e.g. specific strains of *Lactobacillus*

*bulgaricus, Lactobacillus lactis, Lactobacillus helveticus, Lactobacillus amylovorus* etc) cannot be transformed, or with great difficulty only, by any of the commonly used procedures. The invention provides a solution for these species.

The present invention provides a novel method for electroporation of recalcitrant bacteria of the genus *Bifidobacterium*. However, the novel method can, in principle, be applied to any bacterial strain that cannot be transformed by any of the commonly used transformation procedures mentioned above.

Description of the prior art

Bifidobacteria were first described in 1900 by Tissier as *Bacillus bifidus* (Tissier, H. Thesis University of Paris 1900). In 1924 Orla-Jensen recognized the existence of the genus *Bifidobacterium* as a separate taxon and proposed to adopt this name (Orla-Jensen, S. (1924) Lait 4, 468-474). Although there has been much confusion over the years as to the classification of this type of bacteria, there is general agreement now among taxonomists that bifidobacteria should be classified in the genus *Bifidobacterium*, which is now included in the family of *Actinomycetaceae*.

Bifidobacteria are Gram-positive, strictly anaerobic, catalase-negative, fermentative rods, that are often Y- or V-shaped. Traditionally, bifidobacteria are considered as members of the LAB, although this classification is not unanimously accepted. In this report, we will treat bifidobacteria as members of the group of LAB. Bifidobacteria produce acetic acid and lactic acid (3:2) as their major end products. Based on DNA-DNA hybridization measurements and sugar fermentation patterns, 25 species are distinguished within the genus *Bifidobacterium* in the present classification. The GC-content of bifidobacteria is high, between 55 and 64% (Scardovi, V. (1986) In: Bergey's Manual of Systematic Bacteriology. Sneath, H.P. et al, Eds. Vol 2. pp. 1418-1434), in contrast to what is found for other members of LAB, like *Lactobacillus, Lactococcus, Streptococcus, Pediococcus, Leuconostoc, Carnobacterium* and *Enterococcus*, the genome of which has a GC-content varying from 34 to 50%.

The presence of bifidobacteria in the gastro-intestinal tract of human infants and adults has greatly stimulated the interest among bacteriologists and nutritionists. In the intestinal tract of animals and humans bifidobacteria coexist with a large variety of bacteria, most of which are obligate anaerobes. Bifidobacteria are among the most abundant species in the lower small intestine of man and animals. The distal ileum may contain $10^5$-$10^7$ organisms per ml of contents (Gorbach, S.L. et al, (1967) Gastroenterology 53, 856-867; Drasar, B.S. et al, (1969) Gastroenterology 56, 71-79). In the human large intestine bifidobacteria are also prominent, unlike lactobacilli, and are present at concentrations of $10^{10}$ or more per gram contents, constituting 5-10% of total flora in the bowl (Mitsuoka, T. (1992) In: The lactic Acid Bacteria; The Lactic Acid Bacteria in Health and Disease (B.J.B. Wood Ed.) pp 69-114 Elsevier Applied Science).

Studies on the distribution of bifidobacteria in faeces of infants and adults and in the human vagina indicate adaptation of specific species to specific habitats. *B.bifidum* and *B.longum* are predominant species in these three habitats, while other bifidobacterial species are present in only one or two of the habitats (Biavati, B. et al, (1984) Microbiologica 7, 341-345; Biavati, B. (1986) Microbiologica 9, 39-45; Crociani, F. et al, (1973) Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt 1 Orig. Reihe A 223, 298-302). Also species specificity has been observed. *B.magnum* and *B.cuniculi* have been found in rabbit faeces only, while *B.dentium* is consistently found in dental caries of man.

The relationship between bifidobacterial flora and diet in humans of all ages and in animals has been the subject of extensive research (for a review, see Bezkorovainy, A. & Miller-Catchpole, R. (1989) CRC Press, Inc., Boca Raton, Fl). In a comparison of the faecal flora of healthy rural Japanese and healthy urban Canadians, it was found that the flora of individuals eating a Japanese diet contain higher numbers of bifidobacteria than found in people eating a western diet. However, other investigators have reported no effect of the diet on the composition of the intestinal flora and no general

agreement exists as to the effect of the composition of the diet on bifidobacterial flora of humans.

A considerable variety in the structure and composition of the cell wall of bifidobacteria has been reported. A variety of peptidoglycan layers was found in the genus *Bifidobacterium* (Lauer, E. & Kandler, O. (1983) System. Appl. Microbiol. 4, 42-64). In addition, cell walls of bifidobacteria contain significant amounts of polysaccharides, lipoteichoic acids and proteins (Fischer, W. (1987) Eur. J. Biochem. 165, 647-652; Op den Camp, H.J.M. et al, (1984) Biochim. Biophys. Acta 795, 301-313). The presence of a thick (multi-layered) cell wall generally forms a barrier for the uptake of exogenous DNA molecules. The invention relates to a method for weakening the cell wall of bifidobacteria and making the bacteria permeable to nucleic acid (DNA in particular) which avoids the necessity to remove chemicals and/or enzymes which are used in conventional procedures for the introduction of DNA, prior to transformation of the bacteria.

Many endogenous and exogenous factors, like emotional stress, administration of antibiotics and peristaltic disorders may result in an unbalance of the intestinal ecology, which is manifested, amongst others, by a reduction or even disappearance of bifidobacteria. These and other observations suggest that bifidobacteria are important for human (and animal) health. The potential usefulness of bifidobacteria for human and animal health are thought to stem from their capacity to i) contribute to the resistance against bacterial infections (Kaloud, H. & Stogmann, W. (1969) Archieves Kinderheilk. 177, 29-35) by stimulation of the immune response, ii) prevent and treat diarrhea caused by antibiotics (Mayer, J.B. (1966) Mschr Kinderheilk 144, 67-73; Mayer, J.B. (1969) Physikalische Rehabilitation 10, 16-23; Tasvac, B. (1964) Annals Pediatrics 11, 291-307), iii) prevent constipation, geriatric diseases and cancer, iv) stimulate bowel movement by producing lactic acid and acetic acid, and v) produce vitamins, especially B-group vitamins (Mitsuoka, T. (1992) In: The lactic Acid Bacteria; The Lactic Acid Bacteria in Health and Disease (B.J.B.Wood Ed.) pp 69-114 Elsevier Applied Science).

Oral administration of oligosaccharides, in particular fructo-oligosaccharides has been shown to stimulate the growth in the bowl of bifidobacteria. The stimulation of growth is accompanied by a reduction of the faecal pH, of β-glucuronidase and azoreductase activities, and of indole, serum cholesterol and triglyceride levels as wel as blood pressure of elderly people with hyperlipaemia (Tanaka, R. et al (1983) Bifidobacteria Microflora 2, 17-24). Homma (Homma, N. (1988) Bifidobacteria Microflora 7, 35-43) has reported that the presence of bifidobacteria restrained the neo-formation of LDL receptors of human activated T-cells. In rats, *Bifidobacterium* reduced serum cholesterol levels by affecting HMG CoA reductase activity.

Because of the widespread belief that bifidobacteria have health-promoting properties, research is being carried out to identify and apply "factors" that stimulate the growth and metabolism of bifidobacteria. These studies (see for a compilation of research articles, Biavati, B. et al, (1991) In: The prokaryotes (A. Balows et al Eds) pp 816-833) have resulted in the discovery of several growth-promoting compounds, like lactulose, fructose-oligosaccharides, N-acetylglucosamine-containing saccharides (bifidus factor 1), and enzymatically hydrolysed proteins (bifidus factor 2). Modified cow's milk supplemented with different combinations of bifidus factors is marketed as a means to promote the growth of bifidobacteria, in particular for bottle-fed infants.

In western and asiatic countries *Bifidobacterium*-supplemented food is marketed for adults. Since BIOGARDE Company (Munich, Germany) introduced a cultured milk supplemented with bifidobacteria, many other companies in different countries are producing foods to which bifidobacteria have been added as a supplement. Bifidobacteria are also marketed for therapeutic purposes by pharmaceutical companies e.g. for treatment of digestive disturbances in bottle-fed infants, enterocolitis, constipation, disturbed balance of the intestinal flora after treatment with antibiotics, and for the promotion of intestinal peristalsis (Rasic, J.Lj. & Kurmann, J.A. (1983) Bifidobacteria and their role, Birkhäuser Verlag, Basel).

Despite the wealth of data suggesting that bifidobacteria can promote health of human infants and adults, as well as that of animals, a solid scientific basis for many of the claims is lacking. Consequently, more detailed studies are necessary to clarify the therapeutic effects of bifidobacteria, including the role of bifidobacteria in diet. The results of such research are expected to lead to a better use of available bifidobacterial strains, to novel applications of bifidobacteria and to directed improvement of the properties of bifidobacterial strains. The present invention is related amongst other things to the development of a system which allows improvement of the properties of *Bifidobacterium* and of other microorganisms, by genetic engineering, employing recombinant DNA techniques.

Genetic modification of bacteria has been the subject of extensive research over the past decades. Initially most of the research was focussed on the colon bacterium *Escherichia coli* and the soil bacterium *Bacillus subtilis*. More recently, however, methods have been developed which allow the genetic engineering of other bacterial species. To-date systems have been developed for genetic engineering of more than 50 bacterial species.

Foreign DNA can, in principle, be introduced and stably maintained as part of the chromosome or extra-chromosomally, on an autonomously replicating element called episome or plasmid. A gene of interest can be introduced *in vivo* into a bacterium by conjugation, a process which involves the mating of two bacteria and concomittant transfer of DNA from one type of bacterium (donor) to another bacterium (recipient). A second method for *in vivo* introduction of foreign DNA is based on the use of bacteriophages which can transfer genetic information to a recipient bacterium by a process called transduction. Although these techniques have been widely used in the past for a limited number of bacterial species, they have now largely been superseded by the *in vitro* recombinant DNA approach.

Methods for gene-transfer by the recombinant DNA technique are all based on the introduction into an organism (e.g. a bacterium) of a DNA fragment carrying the genetic information for a given trait, which is connected to a carrier DNA molecule,

called vector. In many cases, the vector is a circular molecule which can replicate extra-chromosomally as a multi-copy plasmid in the organism of choice. By introduction of the gene of interest on an autonomously replicating multi-copy vector, the copy number of the gene is increased which may lead to a considerable increase of the product synthesized from said gene.

Because the uptake of DNA molecules by bacteria occurs under natural conditions (the environment) or laboratory conditions (e.g. shakeflask or fermentor) with exceedingly low frequencies only, if at all, methods have been devised to improve the uptake and stable maintenance of exogenous DNA.

Initially all protocols for the uptake of foreign DNA by bacteria were based on the use of protoplasted bacteria or of so-called competent bacteria. The use of competent cells of bacteria is restricted to very few bacterial species, and cannot be employed for LAB (Mercenier, A. & Chassy, B.M. (1988) Biochimie 70, 503-517). Protoplasted (or spheroplasted) bacteria are bacteria from which the outer cell wall has been (partially) removed, in many cases by treatment with specific enzymes, sometimes in the presence of chelating agents. After the introduction of DNA, the protoplasted or spheroplasted bacteria have to regenerate, in order to be able to multiply in liquid medium or on solidified agar-containing medium, and to form a colony on an agar plate. The process of protoplast formation and bacterial regeneration is often difficult to reproduce, and is time consuming. The present invention is also related to a method of introducing DNA without the need of (partially) removing the bacterial cell wall by enzymatic and/or chemical treatment and the subsequent regeneration of protoplasted cells.

A break-through in the development of gene-transfer systems for LAB was realized by Chassy and Flickinger (Chassy, B.M. & Flickinger, J.L. (1987) FEMS Microbiol. Lett. 44, 173-177) who demonstrated the successful use of electroporation to transform *Lactobacillus casei*. Electroporation involves a high-voltage electric discharge through a suspension of cells to induce transient 'pores' in the cell membrane through which DNA can enter the bacterium. Transformation of LAB strains with plasmid DNA by electroporation has been reported for *Lactococcus lactis*

(Harlander, S. (1987) In: J.J. Ferretti & R.C. Curtiss (Eds, Streptococcal Genetics pp 229-233. Washington DC: A.S.M. Publications), *L.casei* (Chassy, B.M. & Flickinger, J.L. (1987) FEMS Microbiol. Lett. 44, 173-177), *L. plantarum* and *L.pentosus* (Posno, M. et al, (1991) Appl. Environm. Microbiol. 57, 1822-1828), *Leuconostoc paramesenteroides* (David, S. et al, (1989) Appl. Environm. Microbiol. 55, 1483-1489), and *St. thermophilus* (Mercenier, A. (1990) FEMS Microbiol Rev. 87, 61-78). For a review, see Chassy, B.M. et al, (1987) Trends Biotechn. 6, 303-309; Trevors, J.T. et al, (1992) In: D.C. Chang, B.M. Chassy, J.A. Saunders & A.E. Sowers (Eds) Guide to Electroporation and Electrofusion pp 265-290 Academic Press.

The majority of species of Gram-negative bacteria as well as many strains of Gram-positive bacteria have been successfully transformed by electroporation. Electroporation replaces the tedious, time-consuming and frequently unreliable protoplast transformation techniques that had taken years to develop for the lactic acid bacteria (Kondo, J.K. & McKay, L.L. (1982) Appl. Environm. Microbiol. 43, 1213-1215; Mercenier, A. & Chassy, B.M. (1988) Biochimie 70, 503-517). However, several factors may influence the outcome of electroporation experiments. The dense Gram-positive cell wall may present a barrier to the entry of exogenous nucleic acid in some strains. This possibility is suggested by the observation that the transformation efficiency of *Listeria monocytogenes* can be raised more than 200-fold by incorporation of low concentrations of the cell wall synthesis inhibitor, penicillin G, in the growth medium (Park, S.F. & Stewart, G.S.A.B. (1990) Gene 94, 129-132). The incorporation of glycine, an agent which affects cell wall biosynthesis, can also lead to increases in electroporation efficiency (Dunny, G.H. et al, (1991) Appl. Environm. Microbiol. 57, 1194-1201). In addition, it has been observed that treatment of cells with muralytic enzymes prior to electroporation of lactococci can be used to raise the transformation frequency (Powell, I.B. et al, (1988) Appl. Environm. Microbiol. 54, 655-660). It is also possible that incompatibility between transforming vectors and resident plasmids may lower frequencies of transformation as was demonstrated for different *Lactobacillus* species (Posno, M. et

al, (1991) Appl. Environm. Microbiol. 57, 1822-1828). Other possible barriers for transformation include the presence in the recipient organism of a system for restriction/modification of DNA, for non-specific nucleases, non-expressed selection marker genes, non-expressed replication-essential functions, and interference with host-essential functions.

In recent years improvements of the method have been achieved by a systematic study of the effects of i) growth conditions of the bacteria, ii) the composition of the electroporation solution, iii) pulse conditions, such as field strength and resistance, iv) the size, concentration and purity of DNA used for transformation, v) the electroporation apparatus, vi) choice of selection markers and conditions used to select for transformants, and vii) choice of plasmid vectors. In general, the conditions for an efficient transformation of a particular strain have to be optimized, as each strain shows specific "transformation characteristics". Large differences in transformation frequency are often observed, even between closely related strains.

As stated, the choice of plasmids to be used for transformation of the selected microorganism is very important.

The present invention provides elegant selection criteria for suitable plasmids and additionally provides very suitable plasmids for such transformations.

Plasmids appear to be present ubiquitously in bifidobacteria. Plasmids were found in 70% of the strains of *B.longum*, the predominant species in the human intestine, in *B.globosum* (22% of strains), the most common species in animals, and in 67% of the *B.indicum* strains, a species exclusively found in asiatic honybee hind-guts. Interestingly, strains of *B.infantis*, which is the species most closely related to *B.longum*, do not harbour plasmids, even when the two species were isolated from the same specimen.

If a plasmid-derived vector is to be used for the introduction of exogenous DNA into *Bifidobacterium*, such a plasmid has to be able to replicate in bifidobacteria. In principle, three types of plasmid may be used for this purpose.

i) A vector which is based on a replicon derived from *Bifidobacterium*. Such a vector is likely to be replicated in the host organism, provided that no essential function has been demolished by the introduction of DNA sequences, such as for example a selective marker, upon construction of the vector.

Bifidobacteria (and other LAB's like e.g. *Lactobacillus delbrueckii* subsp. *bulgaricus*) are considered as GRAS (Generally Recognized As Safe) organisms that can be safely used in human and animal food for the fermentation and/or preservation of food, or as food additives. If a genetically engineered microorganism is to be used for such purposes, regulatory authorities are likely to request a full description of the genetic material used for modification of the bacteria. It is also to be expected that acceptance of a modified organism will be enhanced by making use of a vector containing genes and other DNA sequences that already exist in the host bacterium, or are derived from sources that are evolutionary closely related, and/or from an other GRAS organism.

ii) A plasmid derived from a bacterium which is evolutionary closely related. Many plasmids can be horizontally transfered from one organism to another indicating that elements involved in plasmid replication can be functional in more than one organism (Projan, S. & Novick, R. (1988) Plasmid 19, 203-221; Pouwels, P.H. et al, (1993) Mol. Gen. Genet. 242, 614-622). Bifidobacteria belong to a subclass of the Gram-positive bacteria, the genome of which is very GC-rich. Other genera of this group include *Mycobacterium*, *Corynebacterium* and *Streptomyces*. A computer-assisted analysis of plasmids pAL5000 from *Mycobacterium fortuitum* (Rauzier, R. et al (1988) Gene 71, 315-321) and pXZ10142 from *Corynebacterium glutamicum* (X72691 EMBL-GenBank) shows that considerable similarity exists between these plasmids. In particular, functions involved in replication are strikingly similar. These results suggest that such plasmids can replicate in related organisms, including *Bifidobacterium*.

iii) There exists a plethora of plasmids, mostly derived from Gram-positive bacteria that replicate by a so-called rolling circle replication (RCR) mechanism. Typical examples of RCR-type plasmids are *Staphylococcus aureus* plasmids pC194 and

pE194, and derivatives (for a review, see Gruss, S. & Ehrlich, S.D. (1989) Microbiol. Rev. 53, 231-241) and the *Lactobacillus* plasmids pLP825 and pLPE323, and derivatives (for a review, see Pouwels, P.H. & Leer, R.J. Antonie van Leeuwenhoek (1993) 64, 85-107). Some of these plasmids can replicate in a wide variety of Gram-positive, and occasionally even in Gram-negative bacteria (del-Solar, G. et al (1993) Mol. Microbiol. 5, 789-796). RCR-type of plasmids has also been found to replicate in *Streptomyces lividans* (Gruss, S. & Ehrlich, S.D. (1989) Microbiol. Rev. 53, 231-241), an organism which belongs to the same group of bacteria as *Bifidobacterium*. The present invention relates to the use of all three types of plasmid-derived vectors for introduction of exogenous DNA into *Bifidobacterium*.

Plasmids replicating by the same mechanism, e.g. a RCR-type mechanism, usually are found to be incompatible if their replication elements are evolutionary related (Novick, R. Microbiol. Rev. 51, 381-395). In other words, two plasmids that replicate by the same mechanism cannot co-exist in the same bacterium, unless a selection pressure is exerted to maintain the plasmids. Since at present nothing is known about the mode of replication of bifidobacterial DNA or of plasmid DNA, it is difficult to predict whether or not a transforming plasmid will be compatible with an endogenous plasmid. The invention comprises a method which avoids possible complications of incompatibility by using bifidobacterial strains carrying no endogenous plasmids.

A typical electroporation protocol which was developed for *Lactobacillus pentosus* but also appeared to be applicable (without further optimization of the procedure for specific strains) to other *Lactobacillus* strains involved the following steps. An overnight culture of bacteria is diluted (50-fold) in MRS broth with 1% glycine or 0.2M D,L-threonine and incubated without further shaking at 37°C. Cells are harvested in mid-log phase, chilled on ice, and washed twice with 5mM sodium phosphate (pH 7.4) - 1mM $MgCl_2$. Cells are resuspended in 1/100th of the original volume of ice-cold electroporation buffer (0.9M sucrose, 15mM sodium-phosphate buffer (pH 7.4), and 3mM $MgCl_2$, at a density of approximately $10^{10}$ CFU/ml. Plasmid DNA (5 µg) is mixed with 50 µl of the cell suspension in an Electroporation

Apparatus and subjected to an electric pulse. Following the pulse, the cell suspension is directly diluted with 450 µl of MRS broth and incubated for 1.5 h at 37°C to allow expression of the antibiotic resistance marker. Depending on the strain, transformed bacteria can be directly selected on MRS agar plates containing an appropriate concentration of the antibiotic, or must first be incubated on agar plates containing sub-inhibitory concentrations (e.g. 0.5 µg/ml) of the antibiotic, after which they can be selected by replica plating at the selective (e.g. 5.0 µg/ml) concentration of the antibiotic. Transformants generally are visible after 24 to 48 h of anaerobic incubation at 37°C.

Although the electroporation technique has proven to be widely applicable and can be used to transform bacterial strains from several LAB genera, like *Lactococcus, Pediococcus, Lactobacillus* and *Enterococcus,* some strains of *Lactobacillus* and all *Bifidobacterium* strains have proven sofar to be refractory to efficient and reproducible transformation. This holds in particular for some *Lactobacillus* strains used in dairy fermentation and for some *Lactobacillus* strains originating from the gastro-intestinal tract of man or animals. Despite extensive research in various renowned research laboratories, all attempts to transform *Lactobacillus delbrueckii* subsp. *bulgaricus* or *Lactobacillus delbrueckii* subsp. *lactis* have been unsuccesful. In one report in which the transformation of *L. delbreuckii* subsp. *bulgaricus* was described use was made of a vector derived from *L. delbreuckii* subsp. *bulgaricus* and a mutant strain deficient in restriction/modification (Sasaki, T. et al, FEMS Microbiol Rev. (1993) P8). Of all *Lactobacillus helveticus* strains tested sofar, very few strain (e.g. CNRZ 32) have been shown to be efficiently and reproducibly transformable by electroporation (see e.g. Bhowmik, T. & Steele, J. (1993) J. Gen. Microbiol. 139, 1433-1439).

Over the past few years a considerable amount of research has been carried out in several industrial and academic laboratories to develop a transformation procedure for bifidobacteria. However, as yet without any noticeable success. In a recent patent application (EP 0 319 690 A1) transformation of *Bifido-*

*bacterium longum* strain 108A with plasmid DNA has been claimed. The plasmid used in these studies, pAMβ1, originates from *Enterococcus faecalis* and carries an erythromycin resistance gene. In one experiment described in EP 0 319 690 A1, 11 erythromycin resistant ($Ery^R$) colonies were obtained after three days of incubation when pAMβ1 had been used for transformation, whereas one $Ery^R$ colony was found when DNA was not added. In the same experiment 23 $Ery^R$ colonies were observed after 7 days of incubation, while 3 $Ery^R$ colonies were found in the absence of added DNA. In a second experiment, described in the same report, an even lower number of $Ery^R$ transformants was found. As the presence of pAMβ1 has not been demonstrated in any of the $Ery^R$ colonies obtained, no proof for genuine genetic transformation of *Bifidobacterium* is given in the report. In addition, the frequency with which $Ery^R$ colonies were obtained was only about 3-6 CFU/μg pAMβ1. Such a low frequency of transformation, if it had occurred, would preclude transformation of bifidobacteria with ligated DNA or with non-autonomously replicating DNA.

Summary of the invention

It is an objective of the present invention to provide a novel process for transformation of microorganisms, more specifically of Lactic Acid Bacteria, more specifically bacteria from the genera *Bifidobacterium* and *Lactobacillus*, that cannot, or only very inefficiently and/or irreproducibly, be transformed with any of the currently known methods.

It is a further objective to provide novel and useful transformants of various *Bifidobacterium* species, such as for example, *Bifidobacterium animalis*, *Bifidobacterium bifidum* and *Bifidobacterium longum*, and of *Lactobacillus bulgaricus*, *Lactobacillus helveticus* and species from the *Lactobacillus acidophilus* groups A and B (i.e. *L.acidophilus*, *L.crispatus*, *L.amylovorus*, *L.gallinarum*, *L.gasseri* and *L.johnsonii*).

It is a further objective to provide transformants of said species containing re-introduced engineered genes originating from the same or closely related species, or from a different host, and in which such genes are present on a multi-copy plasmid vector.

It is a further objective of the present invention to provide transformed microorganisms, more specifically transformed bacteria of the genera *Bifidobacterium* or *Lactobacillus*, prepared by the present invention, which contain an easily selectable phenotype which allows them to be readily differentiated from untransformed microorganisms. In addition, said transformed bacteria may contain foreign DNA conferring modified properties to the bacteria.

In a process of the present invention, *Bifidobacterium* species are transformed with a plasmid-derived vector which contains a selectable marker, wherein said vector is able to autonomously replicate in said organisms, but is not found in said organisms prior to transformation.

The vector may also contain other foreign DNA sequences which will modify or enhance expression of foreign DNA.

The invention provides a method of introducing nucleic acid into a microorganism, comprising inducing limited autolysis of the microorganism to improve the permeability of the cell wall of the microorganism for said nucleic acid, contacting the microorganism directly or indirectly with said nucleic acid to transform the microorganism and culturing the resulting transformants. In particular, the invention provides a method for introducing at least one nucleic acid into a microorganism comprising the steps of

a) treating the cell wall of the microorganism to become less impermeable to said nucleic acid, by resuspending these microorganisms in so-called electroporation buffer,

b) contacting the microorganism indirectly or directly with the nucleic acid to be introduced, and

c) culturing the resulting transformants,

whereby step a) is carried out by inducing limited autolysis of the microorganism.

The invention further provides a method as defined above, further comprising subjecting the microorganism to high voltage electric discharge. In particular, the invention provides a method for introducing at least one nucleic acid into a microorganism comprising the steps of

a) treating the cell wall of the microorganism to become less impermeable to said nucleic acid, by resuspending these micro-organisms in so-called electroporation buffer,
b) subjecting the microorganism to a high voltage electric discharge,
c) contacting the microorganism indirectly or directly with the nucleic acid to be introduced, and
d) culturing the resulting transformants,
whereby step a) is carried out by inducing limited autolysis of the microorganism.

In a preferred embodiment the invention provides a method for introducing at least one nucleic acid into a lactic acid bacterium comprising the steps of
a) treating the bacterial cell wall to become more permeable to said nucleic acid,
b) subjecting the lactid acid bacterium to a high voltage electric discharge,
c) contacting the bacterium with the nucleic acid to be introduced, and
d) culturing the resulting transformants,
whereby step a) is carried out by inducing limited autolysis of the lactic acid bacterium.

According to the subject invention, the microorganism is preferably kept in electroporation buffer to induce limited autolysis of the microorganism. Preferably, said electroporation buffer has low molarity and contains an osmotic stabilizer. In a preferred embodiment, said osmotic stabilizer is selected from the group consisting of sucrose, sorbitol, mannitol, glycerol and polyethyleneglycol. Generally, said buffer has a salt concentration of below 10mM, preferably of from about 0.1 to about 2mM, most preferably of from about 0.5 to about 1mM. Preferably, said limited autolysis is carried out at a pH of from about 4 to about 8; at a temperature of from -196°C up to 37°C, preferably at about 0-37°C, more preferably at about 0-10°C, for a time period of from 10 min to 100 hours, preferably from about 1 to about 30 hours.

According to a preferred embodiment of the invention, the microorganism is a recalcitrant. Preferably, the microorganism

is a lactic acid bacterium, in particular a recalcitrant lactic acid bacterium. Most preferably, the bacterium to be transformed by the method of this invention is a *Bifidobacterium* species.

It is preferred that the CG content of the nucleic acid to be introduced corresponds with the CG content of the bacterium into which it is to be introduced. The words "nucleic acid to be introduced" refer to all nucleic acid to be introduced and maintained in the recipient bacterium, i.e. both vector nucleic acid and insert nucleic acid. Furthermore, it is preferred that at least a significant part of the nucleic acid to be introduced is evolutionary related to the microorganism into which it is to be introduced, and preferred that the nucleic acid comprises at least a part carrying the genetic information for providing the microorganism with an improved property. In particular, the nucleic acid comprises at least a part encoding a protein to be expressed by the microorganism.

This invention also provides a transformed recalcitrant microorganism obtainable by any one of the above methods of the invention, in particular a transformed recalcitrant lactic acid bacterium, more preferably a transformed recalcitrant *Bifidobacterium* or *Lactobacillus* species.

The invention also embraces the use of such a transformed recalcitrant microorganism, in particular in the production or formulation of a food product, preferably a dairy product.

Detailed description of the invention

It has been shown, as will be clear from the examples described hereinbelow that by subjecting the microorganisms to a relatively mild autolysis, they can be efficiently and reproducibly transformed.

In a preferred embodiment of a process according to the present invention, bacteria of the genus *Bifidobacterium* are transformed with plasmid vector DNA. To increase the likelihood that the transforming vector is capable of replicating in the recipient host bacterium, plasmid DNA is used that originates from *Bifidobacterium*, more specifically from *Bifidobacterium longum*. To avoid possible complications due to incompatibility,

a strain of *Bifidobacterium animalis* is used harbouring no detectable endogenous plasmids.

In another preferred embodiment of the invention, the technique of electroporation is used to introduce foreign DNA into bacteria, as this technique has proved most reliable and efficient for the introduction of DNA into other types of bacteria. To avoid possible complications which are inherent in regeneration of bacteria that have been exposed to chemicals (e.g. penicillin) and/or enzymes for protoplastation or spheroplastation, bacteria are used that are fragilized by limited autolytic treatment. This mild autolytic treatment not only obviates the need of irreproducible and time-consuming regeneration often seen after protoplastation, but also allows to introduce foreign DNA by means of electroporation without further treatment (removal of protoplasting enzymes like lysozyme, mutanolysin and/or chemicals such as penicillin). In this way the process of transformation is made better reproducible and less time consuming.

Autolysis of lactobacilli has been proposed as a method to rapidly isolate cellular components from *Lactobacillus delbrueckii* subsp. *bulgaricus* (Sasaki, T. et al, (1987) FEMS Microbiol. Rev. D18). The method described by Sasaki et al (1987) involves the incubation of bacteria in the presence of 0.6M citrate at 45°C for 30-150 min. Since the presence of high concentrations of salt in the electroporation buffer, as for example 0.6M citrate, precludes electroporation of bacteria, the preferred method described above used in the present invention involves autolysis of the bacteria at a concentration of buffer of low molarity such as 1mM, in the presence of an osmotic stabilizer. This allows to subject the bacteria directly to an electric pulse, after addition of a sample of DNA, without any further treatment of the bacteria. In the examples 0.5M sucrose is used as an osmotic stabilizer, but other osmotic stabilizers which are commonly used by people skilled in the art, such as e.g. sorbitol, mannitol, PEG may be used as well. As buffers for autolysis are used in the examples citrate buffer pH 5, citrate buffer pH 6, phosphate buffer pH 4.5, phosphate buffer pH 6, Hepes buffer pH 6 and Hepes buffer pH 7.4. However, other

compounds which buffer in the pH range from 4 to 8, such as for example cacodylate, TRIS, malate buffer can be used as well for the autolysis procedure. As stated, the use of buffers for autolysis at concentrations with low molarity (such as between about 0.1 and 2mM) is to be preferred.

Higher buffer concentrations such as e.g. from about 1 or 2 up to about 500mM (or even higher) will also yield "autolysing cells". But under these conditions, a washing step may have to be included. Electroporation in the presence of elevated buffer concentrations will alter the field strength conditions between the electrodes of the electroporator in such a way that the transformation efficiency will be reduced, or that no introduction of DNA will occur. High buffer concentrations (> 10mM) can also damage the electroporation cuvettes. Washing of autolysing cells obtained after incubation in elevated buffer concentrations can also damage these fragilized cells, resulting in a decrease of the efficiency of transformation.

In a following preferred embodiment of the invention limited autolysis leading to electroporation-permeable bacteria is carried out at low temperatures (e.g by storing the bacteria at temperatures between -196°C and 37°C), preferably 4°C (e.g. by placing the bacteria resuspended in 1mM buffer in a refrigerator at 4°C) for a period of 0-25 h, or longer depending on the bacterial strains. This treatment causes no perceptible loss of viability or visible changes of the morphology of the bacteria.

In a following preferred embodiment of the invention, *Bifidobacterium* bacteria are first cultivated overnight at 37°C in MRS broth (Difco; de Man, J.C. et al, (1960) J. Appl. Bacteriol. 23, 130-135) supplemented with 0.05% cysteine and 0.5M sucrose. Sucrose is autoclaved separately and then added to autoclaved MRS broth. Subsequently, bacteria are diluted, e.g. twentyfive-fold (20 ml overnight culture in 500 ml) of the same medium and incubated at 37°C for several hours. After incubation till a density at 695 nm is reached of e.g. about 0.2, bacteria are chilled on ice, collected by centrifugation at 0-4°C (15 min at 2100 x g) and washed twice with ice-cold 0.5M sucrose. After collecting the bacteria by centrifugation at 0-4°C (15 min at 2100 x g), the pellet of bacteria is resuspensed in 1.8 ml ice-

cold 0.5M sucrose. Three µl of 100mM ammonium citrate buffer, pH 6.0 are added to 300 µl of the bacterial suspension, the mixture gently swirled and placed in a refrigerator for 3 h. Although the above-described procedure is a preferred one, it should be stressed that variations in the composition of the growth medium, growth temperature, growth conditions, washing conditions, centrifugation conditions, etc have proven to yield transformed bacteria. The efficiency of transformation may vary, but can easily be optimized, e.g. by altering the duration of incubation of the bacteria in the presence of the autolysis inducing buffer. A change of the temperature at which autolysis takes place may also affect the transformation efficacy.

In another specific preferred embodiment of the invention, 80 µl of cell suspension obtained as described before, is mixed with 2.5 µl of vector DNA (about 1.5 µg), the mixture is gently swirled and transferred into a pre-cooled Gene Pulser disposable cuvette (inter-electrode distance 0.2 cm). A single pulse of 11000 V/cm is delivered immediately (200 Ω parallel resistor; 25 µF capacitance settings; Gene Pulser and Pulse Controller from Bio Rad). Following the pulse (time constant 4-5 msec), the cell suspension is immediately diluted in 800 µl of MRS broth supplemented with 0.05% cysteine and 0.5M sucrose. After 2.5 h of incubation at 37°C, to allow expression of the antibiotic resistance marker, bacteria are plated on selective medium agar (1.4% Daishin agar) plates (MRS + 0.05% cysteine + 0.5M sucrose, plus an appropriate concentration of antibiotic). Transformants are visible after 2-3 days of anaerobic incubation at 37°C. It should be emphasized that, depending on the *Bifidobacterium* strain used, other conditions of electroporation (e.g. a higher or lower voltage), and of growth of the bacteria following electroporation, may be needed to obtain optimal results.

In a following preferred embodiment of the present invention, the plasmid vector pDG7 based on the cryptic plasmid pMB1, which originates from *Bifidobacterium longum* B2577 (Matteuzzi, D. (1990) Letters Appl. Microbiol. 11, 220-223; Figure 1A) is used. Plasmid pDG7 contains an ampicillin resistance marker for selection in *E.coli* and a chloramphenicol resistance marker suitable for selection in may different

bacterial species such as *Escherichia coli, Staphylococcus, Bacillus, Lactococcus* and *Lactobacillus*. The latter marker is used for selection of transformants in bifidobacteria, as this marker has proven very effective in selection of transformants in a range of other Gram-positive bacteria. Under the conditions as given in the previous paragraph, $10^3$ to $8x10^4$ chloramphenicol resistant *Bifidobacterium animalis* colonies are found per µg of pDG7 in different experiments. To verify that the chloramphenicol resistant colonies have been genetically transformed, the plasmid DNA content of 12 transformants was analyzed. Transformation was achieved by electroporation of autolysing cells obtained through incubation in different 1mM buffers (e.g. Hepes, pH 6 or 7.4; citrate pH 5 or 6; phosphate pH 4.5 or 6) for 16 hrs at 4°C. As shown in Figure 2, all transformants contain a plasmid of the expected size. The *Bifidobacterium animalis* transformants show the same carbohydrate fermentation profile as untransformed *Bifidobacterium animalis*, when tested in the API CH50L gallery (API System, Montalieu Verce, France). The structure of the plasmid DNA was further analyzed by restriction enzyme analysis and shown to be identical to that of pDG7.

In another specific embodiment of the invention, *Bifidobacterium animalis, Bifidobacterium breve, Bifidobacterium bifidum, Bifidobacterium infantis* and *Bifidobacterium longum* are used as recipient bacteria, and pDG7 as the transforming vector. For each of the strains tested, chloramphenicol resistant colonies are found after electroporation, under the conditions of the experiment given in the previous paragraph. Transformants obtained by this method contain a plasmid having a size and structure indistinguishable from that of pDG7.

In a further embodiment of the invention, *Bifidobacterium animalis* is transformed with plasmids pECM2 and pEBM3 (Fig. 1B), containing the *Escherichia coli* plasmid pSUP102, the resistance markers for kanamycin and chloramphenicol, and a replicon for *Corynebacterium* (Kalinowsky, J. pers. communication). Chloramphenicol resistant transformants obtained after electroporation of *Bifidobacterium animalis* under the conditions of the experi-

ment as indicated in the previous paragraph, contain a plasmid with the same size and structure as pECM2, the starting vector.

In a further embodiment of the invention a vector that can integrate into the *Bifidobacterium* chromosome is used instead of an autonomously replicating vector like pDG7. Such an integration vector will preferably contain a DNA fragment from *Bifidobacterium* of preferably more than 400 nucleotides, to allow homologous recombination at a predetermined site.

In a further embodiment of the invention, integration of the vector containing the DNA fragment of *Bifidobacterium* is facilitated by making use of a vector from which the gene(s) encoding the replication protein(s) but not the origin of replication are left out, and supplying the replication protein(s) encoding gene(s) *in trans* on a compatible plasmid. The absence from the integrant of a full set of replication functions is likely to increase the genetic stability of the recombinant organism.

In a further embodiment of the invention a food-grade selection marker is used instead of an antibiotic resistance marker. As food-grade markers may be used, e.g. genes from GRAS organisms, like *Bifidobacterium, Lactococcus* and *Lactobacillus* that can confer a dominant selectable phenotype, such as those coding for enzymes involved in (poly)saccharide metabolism (lactose, xylose, starch, inulin) or genes encoding proteins that render the bacterium resistant towards specific drugs like trimetoprim.

The microorganisms or transformants obtainable by the methods according to the invention can basically be used in all applications of the untransformed organisms. When a gene encoding a useful homologous or heterologous protein is introduced into the microorganism, the microorganism can of course be used for the production of said protein. The many applications of the microorganisms are of course clear to the man skilled in the art now that a method for transforming them has been disclosed in the present invention.

Hereunder we give an illustrative list of a number of possibilities, which by no means will be exhaustive.

The method provides an opportunity to genetically modify microorganisms, and more specifically "recalcitrant" bacteria that cannot be transformed with any of the existing transformation procedures.

The possibility to transform bifidobacteria opens new avenues for directed use in the gastro-intestinal tract of bifidobacteria with novel and/or improved properties. Because bifidobacteria are relatively resistant to low pH and to bile acids they can pass the stomach and small intestine without severe loss of viability. They thus serve as ideal vehicles for the introduction of antigens (oral immunization programs), micronutrients like iron, iodine, and vitamins (e.g. group B vitamins or vitamin A).

By providing bifidobacteria with improved capacity to degrade polyfructans, they will be able to autostimulate growth due to the formation of oligofructose residues.

By modification of the carbon fluxes bifidobacteria can be constructed that are able to exclusively produce lactic acid as end product of sugar fermentation, and/or make other desirable compounds.

Short description of the drawings

Figure 1 is a diagrammatic representation of vectors used for transformation.

A) *Bifidobacterium longum* - *Escherichia coli* shuttle vector pDG7; taken from Matteuzzi, D, et al, Lett. Appl. Microbiol. 11, 220-223 (1990);

B) *Corynebacterium glutamicum* - *Escherichia coli* shuttle vectors pECM2/pEBM3; J. Kalinowsky, pers. communication.

Figure 2 shows an analysis of plasmid DNA content of transformed *Bifidobacterium animalis*.

Legend to Figure 2. Miniscreen DNA analysis was carried out as described by Maniatis, T. et al, (1982) Molecular cloning: a laboratory manual. Cold Spring harbor. N.Y., with minor modifications. Five ml of an overnight culture was centrifuged and resupended in 200 µl of 50mM glucose, 10mM EDTA, 25mM TRIS-HCl pH 8, 30 mg/ml lysozyme. After 40 min incubation at 37°C, add 400 µl of 0.2N NaOH in 1% Sodium-dodecyl-sulphate (SDS).

Invert the tube 2-3 times an put on ice for 5 min. Add 300 µl ice-cold solution of 3M potassium-acetate pH 4.8, and vortex holding the tube upside down. Put the tube on ice for 10 min and add 500 µl of a mixture of chloroform and phenol. Vortex and centrifuge for 2 min in an Eppendorf centrifuge. Transfer the supernatant solution to another Eppendorf tube and extract once again with an equal volume of phenol/chloroform. Precipitate DNA by addition to the supernatant of 2 volumes of ethanol and leaving the mixture overnight. Centrifuge for 15 min in an Eppendorf tube. Resuspend the precipitate in 35 µl of water containing 30 µg/ml RNase. Incubate at 37°C for at least 30 min. Apply 15 µl to a gel for gelelectrophoretic analysis.

The lanes show from left to right:

1- 0.5 µg pDG7 (control) isolated from *Escherichia coli*
2- clone from citrate pH 5 (buffer A)
3- clone from citrate pH 5 (buffer A)
4- clone from citrate pH 6 (buffer B)
5- clone from citrate pH 6 (buffer B)
6- clone from phosphate pH 4.5 (buffer C)
7- clone from phosphate pH 4.5 (buffer C)
8- clone from phosphate pH 6 (buffer D)
9- clone from phosphate pH 6 (buffer D)
10- clone from Hepes pH 6 (buffer E)
11- clone from Hepes pH 6 (buffer E)
12- clone from Hepes pH 7.4 (buffer F)
13- clone from Hepes pH 7.4 (buffer F
14- 0.5 µg pDG7 (control) isolated from *Escherichia coli*
15- lambda/EcoRI/HindIII.

Figure 3 shows the effect of temperature and duration of autolysis on transformation efficiency of *Bifidobacterium animalis*. The efficiency is reflected by the number of transformants after introduction of 1 µg pDG7 plasmid vector DNA.

Figure 4 shows the effect of voltage applied on transformation frequency of *Bifidobacterium animalis*. Autolysing cells were mixed with pDG7 DNA in a pre-cooled 0.2 cm cuvette and subjected to an electric pulse as is indicated. Settings of the Gene Pulser and Gene Pulse controller (Bio-rad) were 25 µF and 200 Ω, respectively. The efficiency of transformation is

reflected by the number of transformants obtained per µg of vector DNA.

EXAMPLE 1

Transformation of *Bifidobacterium animalis*

*Bifidobacterium animalis* was transformed by electroporation at two different voltages (6000 and 10000 V/cm) after autolysis of the bacteria in buffers of different composition. Twenty ml of an overnight culture of *Bifidobacterium animalis* in MRS (Difco) containing 0.05% cysteine and 0.5M sucrose is diluted in 500 ml of the same medium and incubated, anaerobically, at 37°C till a density at 695 nm of 0.19 (4-5 h). The bacteria are chilled by placing them in ice-cold water, centrifuged for 15 min at 2100 x g at 4°C, and washed twice with ice-cold 0.5M sucrose. After the second centrifugation step, the bacteria are gently resuspended in 1.8 ml 0.5M sucrose. The resuspended bacteria are divided into 6 equal parts and placed in a 1.5 ml Eppendorf tube. To 300 µl of resuspended bacteria is added 3 µl of the following solutions: A) 100mM ammonium citrate buffer pH 5; B) 100mM ammonium citrate buffer pH 6; C) 100mM potassium phosphate buffer pH 4.5; D) 100mM potassium phosphate buffer pH 6; E) 100mM Hepes buffer, adjusted with NaOH to pH 6; F) 100mM Hepes buffer adjusted with NaOH to pH 7.4. The tubes are placed in a refrigerator. After 16 h, 80 µl of bacterial suspension is mixed, with gentle swirling, with 2.5 µl of pDG7 DNA. The preparation of pDG7 DNA contains 1 µg of pDG7 DNA isolated from *E.coli* and purified by QIAGEN chromatography according to the recommendations of the supplier (DIAGEN) and 0.4 µg pDG7 DNA isolated from *Bifidobacterium animalis* previously transformed with pDG7. The mixture is placed in a pre-cooled 0.2 cm cuvette, and subjected to an electric pulse (10000 V/cm; 200 Ω parallel resistor; 25 µF capacitance settings; GenePulser and PulseController from Bio-Rad). Immediately after the pulse (time constant 4.3-4.4), 800 µl of MRS containing 0.05 % cysteine and 0.5M sucrose are added and the mixture is incubated, for 2.5 h at 37°C, to allow expression of the chloramphenicol resistance marker. Finally, 100 µl of the bacterial suspension is plated on agar-solidified MRS medium

containing 0.05% cysteine, 0.5M sucrose and 10 µg/ml chloramphenicol. Small-size transformants are visible after two days and full-size colonies after three days of anaerobic incubation at 37°C. The results are given in Table 1.

Table 1 Effect of autolysis buffer composition on transformation frequency of *B. animalis* at two different electroporation conditions.

| Buffer | Number of transformants/µg pDG7 DNA 6000 V/cm | 10000 V/cm |
|---|---|---|
| Citrate pH 5 | 65 | 740 |
| Citrate pH 6 | 130 | 2700 |
| Phosphate pH 4.5 | 200 | 940 |
| Phosphate pH 6 | 20 | 500 |
| Hepes pH 6 | 20 | 1700 |
| Hepes pH 7.4 | 0 | 660 |

EXAMPLE 2

Effect of voltage used for electroporation on transformation efficiency

*Bifidobacterium animalis* was transformed with pDG7 DNA. The same protocol was followed as given in EXAMPLE 1 except for a few modifications. After dilution of an overnight culture as given in EXAMPLE 1, bacteria were cultivated in MRS containing 0.05% cysteine and 0.5M sucrose till $OD_{695}$ = 0.22, instead of 0.19, and after centrifugation and washing, were resuspended in 2.0 ml 0.5M sucrose in 1mM ammonium-citrate buffer, pH 6, and sucrose. After overnight storage in the refrigerator, portions of 80 µl of bacterial suspension were mixed with 80 µl of 0.5M sucrose. To 80 µl of the bacterial suspension thus obtained is added 2.5 µl of pDG7 DNA (same as in EXAMPLE 1). Following the pulse (10000 - 12500 V/cm; yielding a time constant of 3.2-3.8 msec), bacteria are treated as in EXAMPLE 1. The results are given in Figure 3.

The presence of pDG7 in the transformants obtained was verified after isolation of DNA from the transformants by agarose-gel electrophoresis. The results are similar as those found for transformants of EXAMPLE 1.

EXAMPLE 3

Effect of temperature and duration of autolysis of the bacteria

The same protocol for autolysis and electroporation was used as in EXAMPLE 1, except that the time and duration of autolysis were varied as indicated in Figure 4 and that the autolysis buffer was 1mM ammonium-citrate pH 6, and sucrose.

EXAMPLE 4

Transformation of different *Bifidobacterium* strains with pDG7

*Bifido bacterium* ATCC 27536, *Bifidobacterium breve* #4, *Bifidobacterium breve* SA, *Bifidobacterium bifidum* U3, *Bifidobacterium bifidum* ATCC 15696, *Bifidobacterium infantis* U1, *Bifidobacterium infantis* ATCC 27920, *Bifidobacterium longum* U2 and *Bifidobacterium longum* Wiesby 2 are used and pDG7 as vector. Bacteria were cultivated as described in EXAMPLE 2 and were harvested when $OD_{695}$ was 0.2-0.25. Harvesting, washing, autolysis buffer and duration of autolysis were carried out using the protocol given in EXAMPLE 2, using an electric pulse of 10000 V/cm (200 Ω parallel resistance and 25 µF). For each of the strains tested, chloramphenicol resistant colonies are found after electroporation, under the conditions of the experiment given in EXAMPLE 2 (Table 2). Transformants obtained in this way contain a plasmid, with a size and structure indistinguishable from that of pDG7 as verified by gelelectrophoretic analysis.

Table 2 Transformation of different *Bifidobacterium* strains with plasmid pDG7.

| Strain | Number of transformants/ µg of pDG7 DNA |
|---|---|
| *B. animalis* ATCC 27536 | $8 \times 10^4$ |
| *B. breve* #4 | $1.3 \times 10^4$ |
| *B. breve* AS | $2 \times 10^2$ |
| *B. bifidum* U3 | $3 \times 10^2$ |
| *B. bifidum* ATCC 15696 | $7.4 \times 10^3$ |
| *B. infantis* U1 | $2.5 \times 10^2$ |
| *B. infantis* ATCC 27920 | $4 \times 10^4$ |
| *B. longum* U2 | $2.6 \times 10^3$ |
| *B. longum* Wiesby 2 | $7 \times 10^4$ |

EXAMPLE 5

Transformation of *Bifidobacterium animalis* with plasmid vectors from *Lactobacillus* and *Lactococcus* commonly used in the transformation of lactobacilli and lactococci

Autolysis and electroporation of the bacteria were as described in EXAMPLE 2 except that *Lactobacillus* plasmids pLP825, pLPE3537, pLPE323 (Posno et al, 1991 Appl. Environm. Microbiol. 57: 1822-1828) and the broad-host range plasmid *Lactococcus* vector pGK12 (Kok et al, 1984 Appl. Environm. Microbiol. 48: 726-731) which replicates in a variety of Gram-positive and Gram-negative bacteria, were used for electroporation of *Bifidobacterium animalis*. Electroporated bacteria were treated as described in EXAMPLE 2. Transformants were selected on plates containing chloramphenicol as selective agent. No transformants were obtained after anaerobic incubation of the selective plates for 2 days at 37°C as described in EXAMPLE 2. After 4-6 days of prolonged incubation occasionally a small number of colonies appeared. When these colonies were analysed for the presence of autonomously replicating plasmid DNA by miniscreen analysis, no plasmid DNA could be observed. In addition, when the chloramphenicol resistant colonies were analysed for the presence of vector DNA sequences by Southern hybridization, no positive signal was observed at either the position of chromosomal DNA or that of autonomously replicating DNA. These results indicate that plasmids containing a *Lactobacillus* or *Lactococcus* replicon cannot be used to transform bifidobacteria.

Claims

1. A method of introducing nucleic acid into a microorganism, comprising inducing limited autolysis of the microorganism to improve the permeability of the cell wall of the microorganism for said nucleic acid, contacting the microorganism directly or indirectly with said nucleic acid to transform the microorganism and culturing the resulting transformants.

2. A method according to claim 1, wherein the microorganism is kept in electroporation buffer to induce limited autolysis of the microorganism.

3. A method according to claim 2, wherein said electroporation buffer has low molarity and contains an osmotic stabilizer.

4. A method according to claim 3, wherein said osmotic stabilizer is selected from the group consisting of sucrose, sorbitol, mannitol, glycerol and polyethyleneglycol.

5. A method according to claim 3 or claim 4, wherein said buffer has a salt concentration of below 10mM, preferably of from about 0.1 to about 2mM, most preferably of from about 0.5 to about 1mM.

6. A method according to any one of the aforegoing claims, wherein said limited autolysis is carried out at a pH of from about 4 to about 8.

7. A method according to any one of the aforegoing claims, wherein said limited autolysis is carried out at a temperature of from -196°C up to 37°C, preferably at about 0-37°C, more preferably at about 0-10°C, for a time period of from 10 minutes to 100 hours, preferably from about 1 to about 30 hours.

8. A method according to any one of the aforegoing claims, further comprising subjecting the microorganism to high voltage electric discharge.

9. A method according to any one of the aforegoing claims, wherein the microorganism is a recalcitrant.

10. A method according to any one of the aforegoing claims, wherein the microorganism is a lactic acid bacterium.

11. A method according to claim 10, wherein the bacterium is a *Bifidobacterium* species.

12. A method according to any one of the aforegoing claims, wherein the CG content of the nucleic acid to be introduced corresponds with the CG content of the bacterium into which it is to be introduced.

13. A method according to any one of the aforegoing claims, wherein at least a significant part of the nucleic acid to be introduced is evolutionary related to the microorganism into which it is to be introduced.

14. A method according to any one of the aforegoing claims, wherein the nucleic acid comprises at least a part carrying the genetic information for providing the microorganism with an improved property.

15. A method according to any one of the aforegoing claims, wherein the nucleic acid comprises at least a part encoding a protein to be expressed by the microorganism.

16. A method according to any one of the aforegoing claims, wherein the nucleic acid is a plasmid comprising elements for replication and/or expression of genes.

17. A method according to claim 16, wherein the plasmid comprises a selection marker.

18. A method according to claim 16 or 17, wherein the plasmid is derived from plasmid pDG7, pECM2 or pEBM3.

19. A transformed recalcitrant microorganism obtainable by a method according to any one of the aforegoing claims.

20. A microorganism according to claim 19 which is a transformed recalcitrant lactic acid bacterium.

21. A microorganism according to claim 19 or claim 20 which is a *Bifidobacterium* species.

22. A microorganism according to claim 21, wherein the *Bifidobacterium* species is selected from the group consisting of *Bifidobacterium animalis, Bifidobacterium breve, Bifidobacterium bifidum, Bifidobacterium infantis* and *Bifidobacterium longum*.

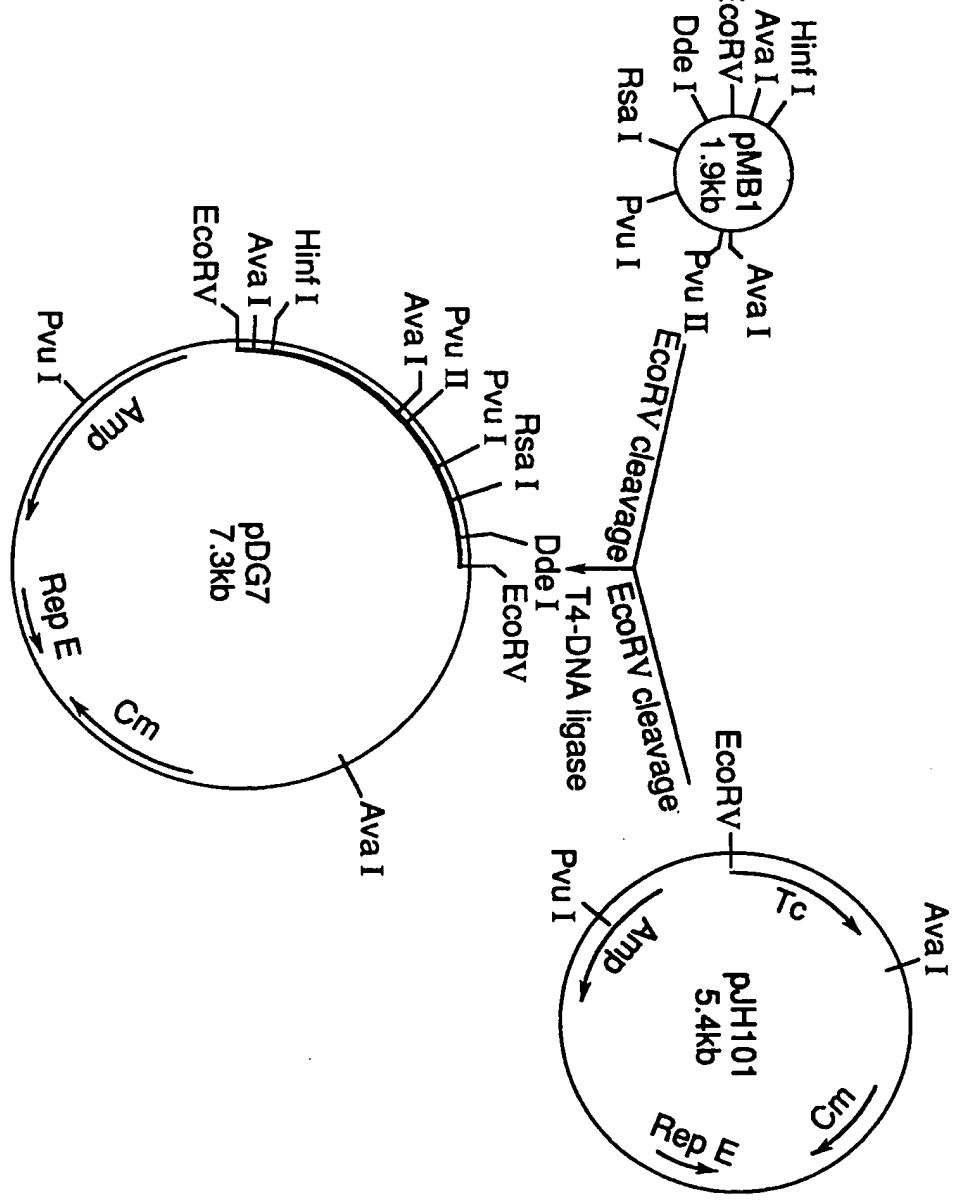
23. A microorganism according to claim 19 or claim 20 which is a *Lactobacillus* species.

24. A microorganism according to claim 23, wherein the *Lactobacillus* species is selected from the group consisting of

*Lactobacillus bulgaricus*, *Lactobacillus helveticus* and a species from the *Lactobacillus acidophilus* groups A and B.

25. Use of a microorganism according to any one of the claims 19-24 in the production or formulation of a food product.

26. Use according to claim 25, wherein the food product is a dairy product.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EcoRV cleavage
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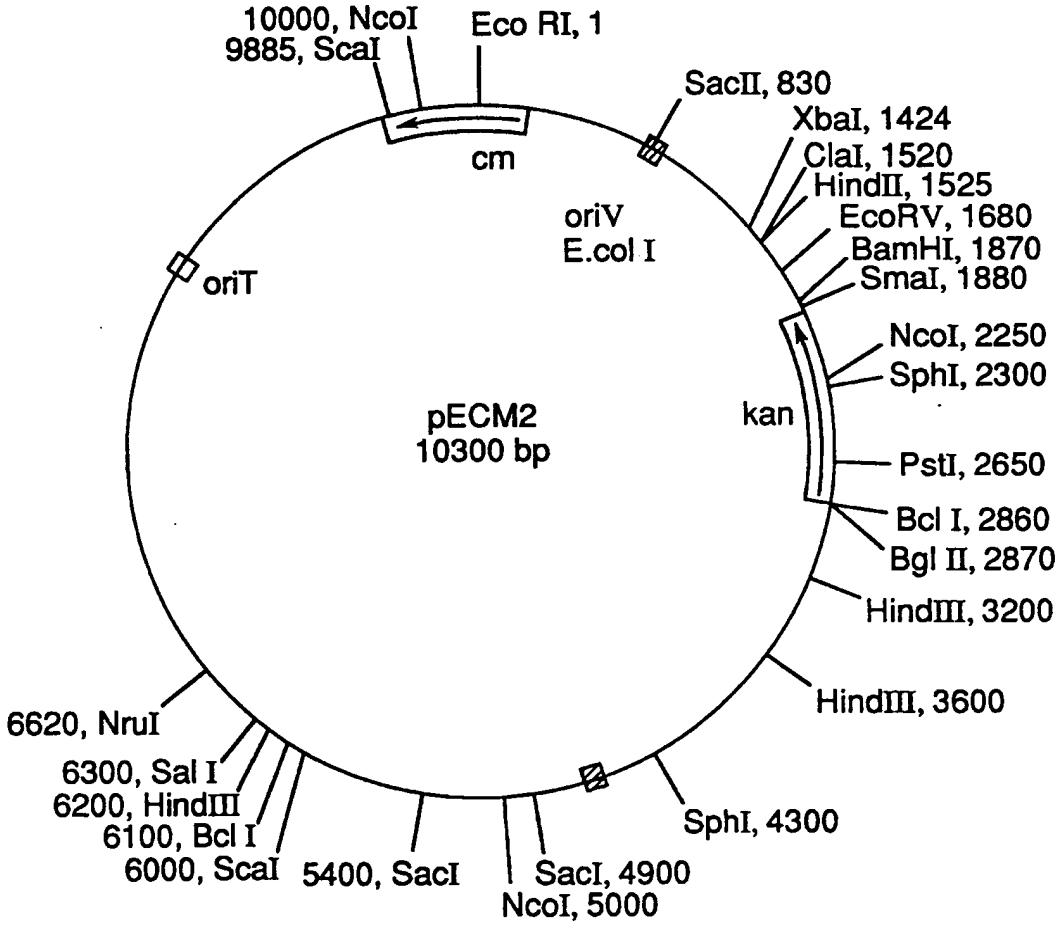
Dde I
EcoRV

FIG. 1A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XbaI, 1424
ClaI, 1520
HindII, 1525
EcoRV, 1680
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SmaI, 1880
NcoI, 2250
SphI, 2300
PstI, 2650
Bcl I, 2860
Bgl II, 2870
HindIII, 3200
HindIII, 3600
SphI, 4300
SacI, 4900
NcoI, 5000
5400, SacI
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oriV
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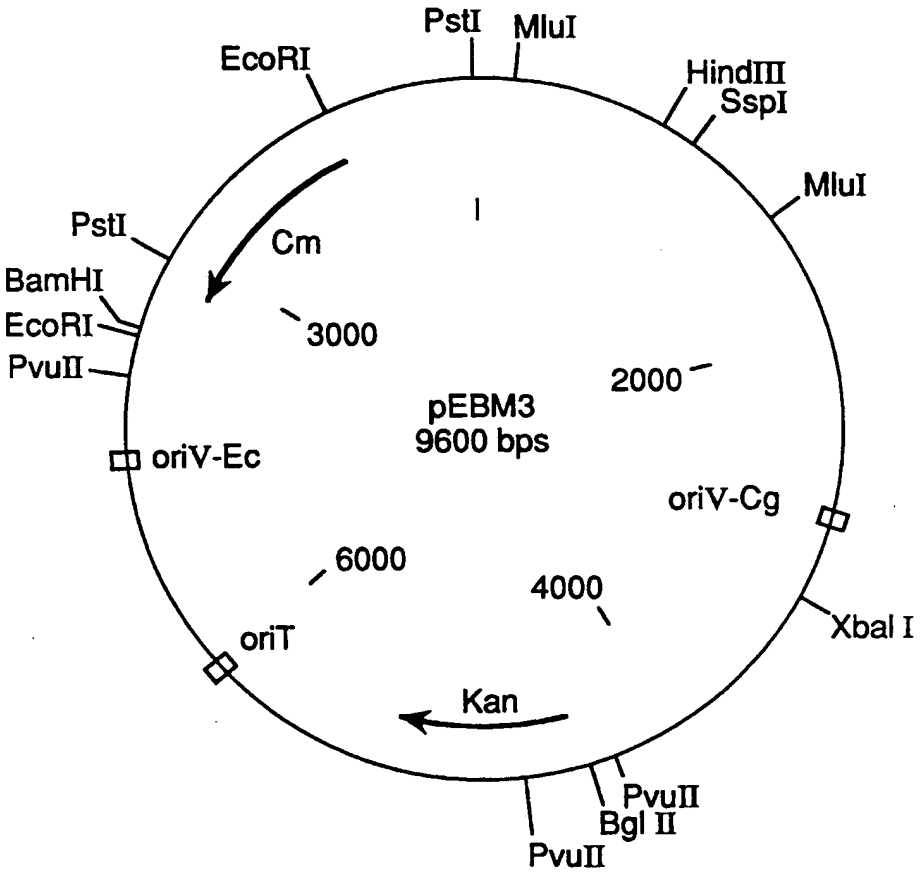
10300 bp
kan

FIG. 1Ba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MluI
PstI
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BamHI
EcoRI
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pEBM3
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oriV-Cg
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4000
XbaI I
oriT
Kan
PvuII
Bgl II
PvuII

FIG. 1Bb

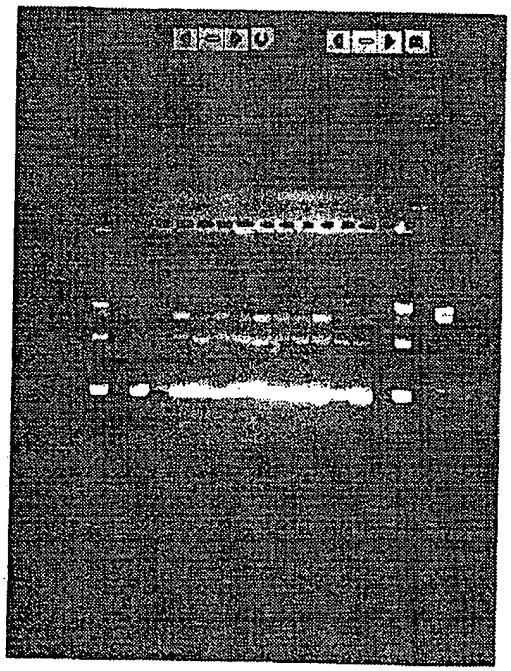

FIG. 2

# INTERNATIONAL SEARCH REPORT

Interr al Application No
PCT/NL 95/00215

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/87 C12N15/74 C12N1/21 A23C9/123 //(C12N1/21, C12R1:225),(C12N1/21,C12R1:01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A23C

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | JOURNAL OF GENERAL MICROBIOLOGY, vol. 139, no. 7, July 1993 pages 1433-1439, T. BHOWMIK ET AL. 'Development of an electroporation procedure for gene disruption in Lactobacillus helveticus CNRZ 32' cited in the application | 1-10, 14-17 |
| X | see abstract<br>see page 1434, left column, paragraph 2<br>see page 1434, right column, paragraph 5<br>see page 1435, right column, paragraph 3<br>see page 1437, left column, paragraph 3<br>---<br>-/-- | 19,20, 23-26 |

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 21 August 1995 | 28.08.95 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax (+31-70) 340-3016 | Montero Lopez, B |

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
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| A | EP-A-0 319 690 (NISSHIN FLOUR MILLING CO., LTD.P) 14 June 1989<br>cited in the application | 1-11,<br>14-17 |
| X | see page 2, line 25 - line 29<br>see page 3, line 16 - line 39<br>see page 3, line 56 - page 4, line 2<br>see page 4, line 9 - line 31; examples 1-15 | 19-26 |
| A | FEMS MICROBIOLOGY LETTERS,<br>vol. 44, no. 2, October 1987<br>pages 173-177,<br>BRUCE M. CHASSY ET AL. 'Transformation of Lactobacillus casei by electroporation'<br>cited in the application | 1-10,<br>14-17 |
| X | see page 173, left column, paragraph 1<br>see page 174, left column, paragraph 2<br>see page 174, right column, paragraph 2 | 19,20 |
| A | WO-A-89 01970 (PLANT GENETIC SYSTEMS, N.V.) 9 March 1989 | 1-10,<br>14-17 |
| X | see page 7, line 31 - page 8, line 4; example 5 | 19,20,23 |
| A | NL-A-8 700 890 (NED. CENTR. ORG. VOOR TOEGEPAST-NATUURWETENSCHAPPELIJK ONDERZOEK) 1 November 1988 | 1-10,<br>13-17 |
| X | see page 5, line 17 - page 8, line 24<br>see page 9, line 8 - line 35<br>see page 10, line 28 - page 11, line 32<br>see page 14, line 12 - line 34<br>see page 14, line 12 - line 34 | 19,20,<br>23-26 |
| A | EP-A-0 262 516 (MILES LABORATORIES, INC.) 6 April 1988<br>see page 3, line 1 - line 19<br>see page 3, line 26 - line 47<br>see page 4, line 20 - line 25 | 1-10,<br>14-17 |
| A | EP-A-0 414 551 (MITSUI TOATSU CHEMICALS, INC.) 27 February 1991<br>see page 2, line 43 - line 46<br>see page 3, line 12 - page 4, line 31 | 1,7,8,<br>14-17 |

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1

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
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information on patent family members

Intern ial Application No
PCT/NL 95/00215

| Patent document cited in search report | Publication date | Patent family member(s) | | Publication date |
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| | | DE-D- | 69016529 | 16-03-95 |
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| | | JP-B- | 7016418 | 01-03-95 |
| | | US-A- | 5124259 | 23-06-92 |